UNIVERSITI PUTRA MALAYSIA

THERMOSTABILITY OF THE RECOMBINANT HAEMAGGLUTININ-NEURAMINIDASE GLYCOPROTEINS OF NEWCASTLE DISEASE VIRUS

TANG YIK KIONG

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By

TANG YIK KIONG

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

July 2003

Dedicated to,

Yik-Shin, Yik-Jia, Parents, Relatives and

My Friends Graduated from Taiwan…

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in the fulfilment of the requirements for the degree of Master of Science

THERMOSTABILITY OF THE RECOMBINANT HAEMAGGLUTININ-NEURAMINIDASE GLYCOPROTEINS OF NEWCASTLE DISEASE VIRUS

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Chairperson : Prof Datin Khatijah Mohd Yusoff, Ph.D.

Faculty : Science and Environmental Studies

The haemagglutinin-neuraminidase (HN) glycoprotein of Newcastle disease virus (NDV) is of primary importance in inducing virus-neutralizing antibodies against viral infection in chicken and has been used in the development of many vaccines. A variant strain of the vaccine strain *V4QUE* known as *V4UPM(HR)* has been developed as a heat stable vaccine for use in the poultry industry in tropical countries such as Malaysia. This protein may also be involved in maintaining heat stability of some vaccine strains. In this study, the HN gene of the heat stable variant NDV strain *V4UPM(HR)* and its parental strain *V4QUE* were cloned and expressed in the Baculovirus Expression Vector System (*BEV*s) and characterized for their heat stability.

The 1.9 kb HN genes of these strains were amplified by RT-PCR from their genomic RNA and unidirectionally cloned into the baculovirus transfer plasmid, *pCR Bac4.8*. These

recombinant baculovirus plasmids were then co-transfected with linearized baculoviral DNA, *Bac-N-Blue*™ DNA into *Spodoptera frugiperda* (*Sf9*) insect cell line. The recombinant baculoviruses which were generated as *recHNV4UPM(HR)* and *recHNV4QUE,* were purified by plaque assay. The respective recombinant HN glycoproteins (*recHNs*) which were expressed in *Sf9* insect cells showed haemagglutination (HA) and neuraminidase (NA) activities as well as haemagglutination inhibition (HI) and haemadsorption activities in serological assays. The HA and NA activities were also detected on the surface and in the cytoplasm of the infected *Sf9* cells. SDS-PAGE and Western blot analysis of the recombinant baculovirus-infected *Sf9* cell lysates detected protein bands of approximately ~74 kDa, which corresponded to the glycosylated HN protein of the virion. These results indicated that the *recHNs* were not only successfully expressed in the *Sf9* cells but they also appeared to be biologically active and functional.

Based on HA activity, the thermostabilities of *recHNV4QUE* and *recHNV4UPM(HR)* together with *recHNAF2240* on HA activity were studied and compared with those of the NDV strains, *V4QUE*, *V4UPM(HR)* and *AF2240*. The latter was earlier showed to be heat stable at 56°C. The results showed that the heat resistance phenotypes of the recombinant baculoviruses were genetically represented identical to NDV individuals in the property of thermostability. NDV heat resistant strains *AF2240*, *V4UPM(HR)* and recombinant baculoviruses *recHNAF2240*, *recHNV4UPM(HR)* were 50% heat inactivated at ~56°C after 4 hours but the parental NDV strain *V4QUE* and baculovirus strain *recHNV4QUE* remained as the temperature sensitive strains.

In addition, the HN genes of the recombinants were sequenced and analyzed by the secondary and three dimensional structure predictions of the computer programs. The roles of individual amino acid residue(s) of the HN protein in thermostability were discussed. The polar/non-polar side chains of the substituted amino acid residues [R32, V413E, N79K in strain *V4UPM(HR)*; E494K in strain *V4UPM*], where the polar side chain could generate the dipole to form the hydrogen bonding with the aqueous environment and increased the thermostability of the protein. The hydrophobic values and secondary structural arrangements of these substituted/deleted amino acid residues [F151Y, I175M and G431S in strain *V4UPM*; A152T, N276K, I280T, V303T, Q372R and Y504H in strain *AF2240*] and [R32, G221, R305, V413E and S584A in strain *V4UPM(HR)*; H87Q and E494K in strain *V4UPM*] formed the secondary structure of the protein for the protein folding events and the protein packing. The thermostable proteins preferred to form the α-helical/β-sheet structures rather than the mixture of the β-sheet/random coil or random coil structures alone at the putative HN domain (399-***GAEG***R-403) where was predicted by the secondary structure arrangement. Besides, the 3D structures of the HN protein supported that any amino acid residue substitutions near the putative active sites (HN domain 399-***GAEG***R-403) could have changed the tertiary structure of the HN protein and these tertiary structure/structural arrangements of the HN protein influenced the heat stability of the HN protein.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia untuk memenuhi keperluan Ijazah Master Sains

KESTABILAN TERMA GLIKOPROTEIN HEMAGLUTININ-NEURAMINIDASE REKOMBINAN DARI VIRUS PENYAKIT NEWCASTLE

Oleh

TANG YIK KIONG

Julai 2003

Pengerusi : Prof Datin Khatijah Mohd Yusoff, Ph.D.

Faculti : Sains dan Pengajian Alam Sekitar

Glikoprotein hemaglutinin-neuraminidase (HN) dari virus penyakit Newcastle (NDV) adalah penting dalam merangsangkan antibodi nyah-virus yang berupaya menentang jangkitan virus dalam ayam, maka ia telah banyak digunakan dalam penghasilan vaksin. Satu strain asing dari strain vaksin *V4QUE* yang dikenali sebagai *V4UPM(HR)* telah di kembangkan sebagai vaksin stabil haba untuk kegunaan industri penternakan di negara tropika misalnya Malaysia. Protein in mungkin juga terlibat dalam mengekalkan kestabilan haba untuk sesetengah strain vaksin. Dalam kajian ini, gen HN dari strain NDV asing *V4UPM(HR)* dan strain induknya *V4QUE* telah diklon dan diekspres dalam Sistem Vektor Ekspresi Baculovirus (*BEV*s) dan telah ditentupastikan kestabilan habanya.

Gen HN yang bersaiz 1.9 kb dari strain NDV tersebut telah digandakan dari RNA genomiknya melalui RT-PCR dan seterusnya diklon secara uni-arah ke dalam plasmid pemindah baculovirus, *pCR Bac4.8*™. Plasmid-plasmid rekombinan baculovirus ini

kemudiannya telah ditranfikasi bersama DNA baculovirus, *Bac-N-Blue*™ yang dilinearkan, ke dalam sel serangga *Spodoptera frugiperda* (*Sf9*). Baculovirus rekombinan yang dinyatakan sebagai *recHNV4UPM(HR)* dan *recHNV4QUE* ini, telah ditulenkan melalui asai plak. Glikoprotein HN rekombinan (*recHN*) yang dihasilkan dalam sel serangga *Sf9* ini menunjukkan aktiviti-aktiviti hemaglutinasi (HA) dan neuraminidase (NA), dan juga aktiviti-aktiviti perencatan hemaglutinasi dan hemaserapan dalam asai-asai serologi. Namum aktiviti-aktiviti HA dan NA ini juga dapat dikesan pada permukaan mahupun dalam sitoplasma sel-sel *Sf9* yang terjangkit. Analisis SDS-PAGE dan Western blot terhadap lisat sel *Sf9* yang dijangkiti baculovirus rekombinan telah menunjukkan jalur protein pada lebih kurang 74 kDa, di mana ia bepadanan dengan protein HN yang berglikolasi dari virus. Keputusan ini menandakan *recHN*s bukan sahaja telah berjaya dihasilkan dalam sel-sel *Sf9*, bahkan ia menampilkan keaktifan dan berfunsi biologinya.

Berdasarkan aktiviti HA, kestabilan terma *recHNV4QUE* dan *recHNV4UPM(HR)* bersama dengan *recHNAF2240* telah dikaji dan dibandingkan dengan strain-strain NDV lain, misalnya *V4QUE*, *V4UPM(HR)* dan *AF2240*. Namun begitu, strain-strain yang kemudian itu menunjukkan bahawa ia mengekalkan kestabilan haba pada suhu ~56°C terlebih dahulu. Keputusan-keputusan menunjukkan fenotip rintangan haba bagi baculovirus-baculovirus rekombinan adalah bersamaan secara genetik dengan individu-individu NDV dari segi sifat kestabilan terma. Strain-Strain rintang haba seperti *AF2240* dan *V4UPM(HR)* serta baculovirus rekombinan *recHNAF2240* dan *recHNV4UPM(HR)* dinyahaktifkan sebanyak 50% pada ~56°C selepas 4 jam. Namun begitu, strain induk NDV

V4QUE dan baculovirus rekombinan strain *recHNV4QUE* tetap merupakan strain sensitif haba.

Gen-gen HN rekombinan ini turut dijujuk dan protein-protein telah dianalisis melalui ramalan struktur sekunder dan tiga dimensi. Peranan-peranan jujukan asid amino individu pada protein HN dalam kestabilan termanya juga dibincangkan. Rantai sisi berkutub/tak berkutub pada asid amino yang digantikan residues [R32, V413E, N79K di strain *V4UPM(HR)*; E494K di strain *V4UPM*], di mana rantai sisi berkutub dapat mewujubkan dwikutub untuk pembentukan ikatan hidrogen dengan persekitaran akueous dan peningkatan kestabilan terma sesuatu protein. Nilai-nilai hidrofobik dan penyusunan struktur sekundernya pada jujukan asid amino terganti [F151Y, I175M, G431S di strain *V4UPM*; A152T, N276K, I280T, V303T, Q372R, Y504H di strain *AF2240*] dan [R32, G221, R305, V413E, S584A di strain *V4UPM(HR)*; H87Q, E494K di strain *V4UPM*] membentuk struktur sekunder protein bagi pelipatan dan penyusunannya. Protein-protein yang stabil haba lebih cenderung untuk membentuk struktur heliks-α/kepingan-β daripada campuran kepingan-β/lingkaran rawak atau struktur lingkaran rawak sahaja pada domain HN anggapan (399-***GAEG***R-403) seperti yang diramalkan menerusi penyusunan struktur sekundernya. Selain itu, struktur 3D protein HN menyokong bahawa sebarang pergantian jujukan asid amino berhampiran dengan tapak domain aktif anggapan (domain HN 399-***GAEG***R-403) mungkin akan mengubah struktur tertiar protein HN dan struktur tertiar/penyusunan struktur protein HN. Ia seterusnya mempengaruhi kestabilan haba pada protein HN.

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I certify that a Supervisory Committee met on 4th August 2003 to conduct the final examination of Tang Yik Kiong on his Master of Science thesis entitled "Thermostability of The Recombinant Haemagglutinin-neuraminidase Glycoproteins of Newcastle Disease Virus" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Supervisory Committee are as follows:

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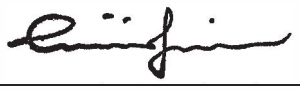

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

TANG YIK KONG

Date: 23 SEP 2003

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LIST OF ABBREVIATIONS

A	
A	Ampere
AcMNPV	Autographa Californica Multiple Nuclear Polyhedrosis Virus
Amp	Ampicillin
α	Alpha
B	
β	Beta
BCP	1-bromo-3-chloropropane
*BEV*s	Baculovirus Expression Vector system
BmNPV	*Bombyx mori* (Silkworm) Nuclear Polyhedrosis Virus
BV	Budded Virus
C	
cDNA	Complementary Deoxyribonucleic Acid
CRBC	Chicken Red Blood Cells
CO_2	Carbon Dioxide
D	
ddNTP	Dideoxynucleotide Triphosphates
DEPC	Diethylpyrocarbonate
dH_2O	Distilled Water
DMF	Dimethylformamide
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
E	
ECV	Extracellular Virus
EDTA	Ethylenediaminetetraacetic Acid Disodium Salt
E. coli	*Escherichia coli*
F	
F	Fusion Protein
Fig.	Figure
FBS	Fetal Bovine Serum
G	
Gal	Glyco
g	Gram
x*g*	x Gravity
H	
h	Hour

HA	Haemagglutinin Activity
HAU	Haemagglutinin Activity Unit
HI	Haemagglutination Inhibition
HN	Haemagglutinin-neuraminidase Protein
HR	Heat Resistance

I

ICTV	The International Committee in the Taxonomy of Viruses

K

KCl_2	Potassium chloride
Kb	Kilobase
kDa	Kilodalton

L

l	liter
ln	Base 2 Logarithms
L	Polymerase Protein

M

M	Matrix Protein
m	Milli
ml	Milli Liter
mM	Milli Molar
MCS	Multiple Cloning Sites
min	Minute
M	Molar
Mr	Molecular Weight

N

NANA	*N*-aceylneuraminic Acid
NA	Neuraminidase Activity
ND	Newcastle Disease
NDV	Newcastle Disease Virus
n	nino
NOVs	Non-occluded Virus
NP	Nucleoprotein

O

ooc^-	Absence of Occlusion Bodies
ooc^+	Presence of Occlusion Bodies
ORF	Open Reading Frame
OV	Occlusion Virus

P

PBS	Phosphate Buffer Saline

PCR	Polymerase Chain Reaction
PIBs	Polyhedral Inclusion Bodies
polh	Polyhedrin Genes of Baculovirus
P	Phosphoprotein
p	pico
PVDF	Polyvinylidene Difluoride Membranes

R

RBC	Red Blood Cells
rec	Recombinant
recHN	Recombinant Haemagglutinin-Neuraminidase Glycoprotein
RE	Restriction Enzymes Analysis
RNA	Ribonucleic Acid
rpm	Round per Minute
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
RT	Room Temperature

S

SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
sec	Second
*Sf-900II*SFM	*Sf-900II* Serum Free Medium
Sf9	*Spodoptera frugiperda*
SFM	Serum Free Medium

T

Taq	Thermus Aquaticus
TAE	Tris-acetate EDTA Buffer
TBE	Tris-boric EDTA Buffer
TE	Tris-base Buffer
Tm	Melting Temperature
Tn	*Trichoplusia ni*

U

µ	micron
UV	Ultra Violet
U	Unit
UPM	Universiti Putra Malaysia

V

v	Volume
V	Volt

W

wt	Wild Type
w	Weight

LIST OF AMINO ACIDS AND ABBREVIATIONS

A	Alanine (Ala)
C	Cysteine (Cys)
D	Aspartic Acid (Asp)
E	Glutamic Acid (Glu)
F	Phenylalanine (Phe)
G	Glycine (Gly)
H	Histidine (His)
I	Isoleucine (Ile)
K	Lysine (Lys)
L	Leucine (Leu)
M	Methionine (Met)
N	Asparagine (Asn)
P	Proline (Pro)
Q	Glutamine (Gln)
R	Arginine (Arg)
S	Serine (Ser)
T	Threonine (Thr)
V	Valine (Val)
W	Tryptophan (Trp)
Y	Tyrosine (Tyr)